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**Primary skeletal muscle cells cultured on gelatin bead  
microcarriers develop structural and biochemical features  
characteristic of adult skeletal muscle<sup>†</sup>**

by

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**Running title:** Primary skeletal myotubes with adult features

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**Abbreviations:** CK, creatine kinase; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; HRP, horseradish peroxidase; Mf, myofibrils; MHC, myosin heavy chain; MHCemb, embryonic MHC; MHCneo, neonatal MHC; Mi, mitochondria; NCS, neonatal calf serum; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline solution; RT, reverse transcription; SEM, scanning electron microscopy; SL, sarcolemma; SR, sarcoplasmic reticulum; TEM, transmission electron microscopy.

## Abstract

A primary skeletal muscle cell culture, in which myoblasts derived from newborn rabbit hindlimb muscles grow on gelatin bead microcarriers in suspension and differentiate into myotubes, has been established previously. In the course of differentiation and beginning spontaneous contractions, these multinucleated myotubes do not detach from their support. Here, we describe the development of the primary myotubes with respect to their ultrastructural differentiation. Scanning electron microscopy reveals that myotubes not only grow around the surface of one carrier bead but also attach themselves to neighbouring carriers, forming bridges between carriers. Transmission electron microscopy demonstrates highly ordered myofibrils, T-tubules and sarcoplasmic reticulum. The functionality of the contractile apparatus is evidenced by contractile activity that occurs spontaneously or can be elicited by electrostimulation. Creatine kinase activity increases steadily until day 20 of culture. Regarding the expression of isoforms of myosin heavy chains (MHC), we could demonstrate that from day 16 on, no non-adult MHC isoform mRNAs are present. Instead, on day 28 the myotubes express predominantly adult fast MHCIIId/x mRNA and protein. This MHC pattern resembles that of fast muscles of adult rabbits. In contrast, primary myotubes grown on matrigel-covered culture dishes express substantial amounts of non-adult MHC protein even on day 21. To conclude, primary myotubes grown on microcarriers in their later stages exhibit many features of adult skeletal muscle and characteristics of fast type II fibers. Thus, the culture represents an excellent model of adult fast skeletal muscle, for example when investigating molecular mechanisms of fast-to-slow fiber type transformation.

**Keywords:** creatine kinase; myofibrils; myosin heavy chain; T-tubules

## 1 Introduction

Differentiated skeletal muscle cells possess an extremely well ordered intracellular organisation, which develops after fusion of myoblasts to multinucleated myotubes. Highly specialized structures comprise the contractile apparatus as well as membranous structures like the sarcoplasmic reticulum (SR) and T-tubules (Eisenberg, 1983; Peachey and Franzini-Armstrong, 1983). Vertebrate skeletal muscle consists of different fiber types, expressing different isoforms of the myosin heavy chain (MHC), which are considered to be a hallmark of fibre type. One slow (type I/ $\beta$ ) and three fast (IIa, IId/x and IIb) fiber types are found in adult skeletal muscle which differ in their contraction speed, strength, and fatigability (Booth and Baldwin, 1996). Transformation of fiber types can occur in response to altered physiological demands (Goldspink, 2002), conferring on skeletal muscle a remarkable plasticity. During development *in vivo*, MHC isoforms are expressed in a distinct sequence, with the expression of non-adult isoforms preceding adult MHC isoforms (Buckingham, 1985; Weydert et al., 1987).

Muscle fiber type-specific gene expression has been examined in human subjects in the context of different exercise regimen, taking muscle biopsies before, after and even during exercise periods (Fluck and Hoppeler, 2003), or in animal models (Pette and Vrbova, 1999; Schiaffino, 2010). Nevertheless, to understand signal transduction pathways and their interactions underlying muscle fiber type-specific gene expression on a molecular level it is necessary to use *in vitro* systems. So far, different approaches have been used to develop a well characterized *in vitro* model, including primary muscle cell cultures prepared from embryonic (Vandenburgh et al., 1991), neonatal (Naumann and Pette, 1994), or adult muscles (Wehrle et al., 1994), and myogenic cell lineages like mouse C2C12 (Zebedin et al., 2004) or rat L8 (Zetser et al., 1999). An important question to be addressed to all these models is whether the cultured myotubes possess quasi-adult properties, especially with

respect to the expression of skeletal muscle-specific genes and differentiation of subcellular structures. Indeed, developmental progression of myosin gene expression, analogous to that seen *in vivo*, has been detected in cultured muscle cells (Silberstein et al., 1986; Weydert et al., 1987). Cultures derived from satellite cells or neonatal myoblasts of different origin as well as cultures based on myogenic cell lines finally develop to express various mixtures of non-adult and adult MHC isoforms (Silberstein et al., 1986; Weydert et al., 1987; Dusterhoft and Pette, 1993; Naumann and Pette, 1994). A simplified but robust method for the isolation of various avian and mammalian muscle satellite cells has been demonstrated recently (Baquero-Perez et al., 2012).

Previously, we have established a primary skeletal muscle culture in which myoblasts derived from newborn rabbit hindlimb muscle grow on microcarriers in suspension and differentiate into myotubes (Kubis et al., 1997). Here, we now describe the development of the primary muscle cell culture with respect to the ultrastructural differentiation of the multinucleated myotubes as well as the developmental pattern of mRNA expression of non-adult MHC isoforms and adult MHCIIId/x. In addition, we have determined the enzymatic activity of creatine kinase as one indicator of muscular differentiation. The data presented here demonstrate that the primary myotubes grown on microcarriers develop ultrastructural components that are surprisingly close to the characteristics of adult muscle cells *in vivo*, such as T-tubules, sarcoplasmic reticulum and highly ordered myofibrils. In addition, we demonstrate complete replacement of non-adult by adult MHC isoform mRNAs during development of the primary myotubes as it is seen in muscle cells *in vivo*.

## **2 Material and methods**

### **2.1 Primary cell culture**

Skeletal muscle cells from hind limbs of newborn New Zealand white rabbits were isolated and separated from fibroblasts as described previously (Kubis et al., 1997). Myoblasts were diluted to a final concentration of  $6.5 \times 10^5$  cells/ml in complete Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Karlsruhe, Germany) supplemented with 10% neonatal calf serum (NCS; Invitrogen). Fifteen ml of this myoblast suspension were transferred to a 75 cm<sup>2</sup> cell culture flask together with 0.04 g gelatin bead microcarriers (CultiSpher-GL; Percell Biolytica, Astorp, Sweden). Cells were grown at 37°C with 8% CO<sub>2</sub> and 90% humidity. On day 2 the medium was changed to 30 ml of Skeletal Muscle Growth Medium with 5% foetal calf serum (FCS) and supplements (PromoCell, Heidelberg, Germany). On day 4, half of the medium was replaced. On day 7, the medium was changed to DMEM with 10% rabbit serum (PAN-Biotech, Aidenbach, Germany). On day 9, the medium was changed to DMEM with 5% NCS. From this day onwards, half of the medium was replaced every second or third day. The cells were grown under continuous shaking on an orbital shaker (~53 rpm).

Alternatively, 5 ml of isolated myoblasts ( $8.5 \times 10^5$  cells/ml DMEM) were seeded on 60 mm cell culture dishes (Nunc, Thermo Fisher Scientific Inc.) coated with matrigel (Collaborative Research, Bedford, MA, U.S.A.). Cells were grown for 21 days at 37°C with 8% CO<sub>2</sub> and 90% humidity. For preparation of matrigel-coated culture dishes, 1 ml of matrigel (4°C) was diluted with DMEM (1:3) and 0.98 ml of the solution was spread on the bottom of the culture dishes at 4°C. The culture dishes were then kept at 37°C for 30 min.

New Zealand white rabbits were purchased via the Animal Facility of Hannover Medical School and experiments were performed as approved by the local authorities (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit/Tierschutz).

## **2.2 Scanning electron microscopy**

Aggregates consisting of myotubes growing on microcarriers were isolated by sedimentation and were pipetted onto collagen-coated glass slides. After incubation at 37°C in 8% CO<sub>2</sub> in air and 95% humidity for 3 hours aggregates were sufficiently attached and slides were washed two times with 0.1 M cacodylate, pH 7.3. Fixation was carried out by rinsing the carriers with 2.5% (v/v) glutardialdehyde in 0.1 M cacodylate buffer and incubating again for 2 hours under the former conditions. Furthermore, the carriers were dehydrated through graded acetone and were dried by the critical point method. The dried carriers were coated with golden-palladium and were then examined with a *PHILLIPS EM 505* scanning electron microscope.

### **2.3 Transmission electron microscopy**

The carriers covered with myotubes were washed with 0.1 M cacodylate buffer, pH 7.3, and were fixed with 2.5% (v/v) glutardialdehyde/2% (w/v) paraformaldehyde in 0.1 M cacodylate buffer for 6 hours. After rinsing with the same buffer carriers were post-fixed with osmium tetroxide (1%) and ferrocyanide (1.5%) in aqua bidest. Microcarriers were dehydrated and then embedded in Epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a *SIEMENS ELMISKOP 101* electron microscope at 80 kV.

### **2.4 Northern Blot analysis**

For isolation of total RNA, cells on microcarriers were washed twice with phosphate buffered saline solution (PBS). Total RNA isolation was performed according to the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987) using the RNAwiz<sup>TM</sup> RNA isolation system (Ambion, Darmstadt, Germany), as described previously (Meissner et al.,



2001). Isolated RNA was separated on 1.2% agarose-formaldehyde gels, transferred to nitrocellulose (Macherey & Nagel, Dueren, Germany), and hybridized with MHC isoform specific cDNA probes from the 3' terminal regions. The probes were labeled with [<sup>32</sup>P]dCTP using random hexamers as primers (Feinberg and Vogelstein, 1983), applying the Prime-a-Gene labelling system (Promega, Mannheim, Germany). For detection of neonatal MHC (MHCneo) mRNA, the 3' terminal 250 bp *Pst*I fragment from mice MHCneo cDNA clone pMHC16.2A (Weydert et al., 1985) was used. Embryonic MHC (MHCemb) mRNA was detected with the 500 bp 3' terminal *Bam*HI/*Eco*RI fragment of the human MHCemb cDNA clone pHEMHC-1 (Eller et al., 1989), and MHCIId/x mRNA with the 3' terminal 579 bp *Pst*I fragment of the rabbit MHCIId/x cDNA clone pMHC24-79 (Maeda et al., 1987), specific for fast MHC isoform IId/x (Uber and Pette, 1993). 18s rRNA was detected with the 5.8 kb Hind III fragment of 18S rDNA clone pXC-1 (Katz et al., 1983).

## **2.5 Semiquantitative reverse transcription (RT)-PCR**

Cells on microcarriers were washed twice with PBS and covered with RNA-Later (Ambion, Darmstadt, Germany) and stored at -20°C as recommended. RNA isolation was carried out later with the RNeasy Kit (Qiagen, Hilden, Germany) including digestion of genomic DNA with RNase-free DNase. The cDNA was synthesized from 1.5 µg of total RNA using Omniscript Reverse Transcriptase (Qiagen) as instructed. Semiquantitative multiplex PCR was performed using the HotStarTaq DNA Polymerase Kit (Qiagen) with primers for MHCIId/x cDNA amplification (accession no. U32574; forward: 5'-TGAAGACTCTGGCCTTCCTC-3', reverse: 5'-TGATCGACCGTACAAAGTGG-3'), and QuantumRNA 18S Internal Standard Primers and Competimers (Ambion) for normalization. The PCR program consisted of cycles of 95°C for 1 min followed by 60°C for 1 min and 72°C for 1 min, after an initial activation of the HotStarTaq at 95°C for 15 min. The

exponential range of PCR amplification and the appropriate ratio of 18S primers to 18S competitors were determined in preliminary experiments. PCR products were separated with polyacrylamide gel electrophoresis (PAGE) (8% total acrylamide-bisacrylamide monomer concentration, 5% crosslinker concentration) using Low DNA Mass Ladder (Invitrogen, Karlsruhe, Germany) as a marker, followed by highly sensitive silver staining (Bassam et al., 1991). Densitometric quantification of bands was performed with an Image Master System (GE Healthcare, Munich, Germany).

## **2.6 Myosin heavy chain electrophoresis**

After centrifugation of cell homogenates at 100,000 x g at 4°C for 1 hour pellets were extracted with 0.6 M KCl, 1mM EGTA, 0.5 mM DTT, 0.5 mM PMSF, 10 mM potassium phosphate, pH 6.8 at 0°C for 30 min. Supernatants of a further centrifugation at 20,000 x g for 20 min were diluted 1:10 (v/v) with ice-cold water and actomyosins were precipitated over night at 0°C. Suspensions were centrifuged again at 20,000 x g for 30 min at 4°C and actomyosin pellets were redissolved with extraction buffer and stored at -80°C. Actomyosin samples were diluted (1:3) with the modified Laemmli sample buffer according to Cannon-Carlson and Tang (Cannon-Carlson and Tang, 1997) and denaturated at 95°C for 5 min. Electrophoresis was performed by the modified method of Kubis and Gros (Kubis and Gros, 1997) using a slab gel (21x16 cm, 0.5 mm gel thickness) with 3.5% stacking gel (pH 6.8), a 6.6% separating gel (pH 8.8) with 5% glycerol, followed by a second stacking gel 6.6% (pH 6.8) with 15% glycerol and a second separating gel 8.8% (pH 8.8) with 30% glycerol. Running conditions were 12 mA/220V at 4°C for 24 hours. After run gels were silver stained according to Heukeshoven and Dernick (Heukeshoven and Dernick, 1985). Quantification of MHC bands was performed using IMAGE MASTER 1D software (Pharmacia).

## 2.7 Western blot analysis

Western blot analysis was performed as described previously (Scharf et al., 2013) using anti-MHCIId/x (Developmental Studies Hybridoma Bank, The University of Iowa, Iowa City, IA, USA) or anti- $\alpha$ -tubulin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibodies. Bound antibodies were detected with anti-mouse IgG conjugated to horseradish peroxidase (HRP) (Promega). Signals were visualized by enhanced chemiluminescence detection.

## 2.8 Enzyme activity measurements

The supernatants of homogenated myotubes were used for determination of enzyme activities after a 100,000 x g centrifugation (see above). Creatine kinase activities were determined using standard optically coupled assays (Bass et al., 1969). Protein concentrations were detected by the method of Oyama and Eagle (Oyama and Eagle, 1956).

### 1.1 2.9 Immunofluorescence studies

#### 1.2

Primary myotubes grown on microcarriers for 14 days were detached from the carriers by incubation with Accutase (Sigma-Aldrich, Taufkirchen, Germany) for 20 minutes. After sedimentation of the microcarriers and centrifugation of the supernatant at 330 x g for 5 min myotubes were resuspended in DMEM/10% NCS. Resuspended cells were then seeded on glass coverslips and cultured for additional two days in DMEM/10% NCS. In a further experiment, 0.1 mM of calcium ionophore A23187 (Sigma-Aldrich) was added to the medium on day 2. After day 2, cells were washed in PBS, fixed with 100% methanol, permeabilized in 0.1% Triton X-100/PBS and washed 3 times with PBS. To prevent non-specific binding, cells were treated for 30 min with 0.2% bovine albumin serum (BSA) and

then incubated for 30 min with primary rabbit polyclonal anti-NFATc1 antibody (H-110; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Subsequently, cells were washed with PBS and incubated with secondary fluorescein isothiocyanate (FITC)-labeled anti-rabbit antibody (Santa Cruz Biotechnology, Inc.). Immunostained myotubes or cells with nuclei stained by the fluorescence DNA marker 4',6-Diamidin-2-phenylindol (DAPI; Sigma) were photographed on an inverted fluorescence microscope (Leica Microsystems, Wetzlar, Germany) at a magnification of x 400.

## **2.10 Statistical analysis**

Results are expressed as means  $\pm$  SD of three or more independent experiments. The statistical significance of differences of these means was estimated by one-way ANOVA followed by the Newman-Keuls multiple comparison test, performed using GraphPad Prism software version 3.00.

## **3 Results**

### **3.1 Electron microscopy reveals structural features of the primary myotubes as found in adult skeletal muscle**

Rabbit primary skeletal muscle cells growing on gelatin bead microcarriers as described previously (Kubis et al., 1997) were investigated by scanning electron microscopy (SEM). Nine days old myotubes on microcarriers exhibited a round shape and were rather short (Figure 1A). After 16 to 20 days of culture the myotubes showed a spindle-like shape with increased length (Figure 1B). Myotubes were found to be attached to single carriers but also to two neighbouring carriers, thereby forming bridges from carrier to carrier and sometimes forming aggregates of more than two microcarriers (Figures 1B and C).

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We next used transmission electron microscopy (TEM) to further investigate the ultrastructural organisation of the primary myotubes growing on microcarriers. On day 9, TEM shows loosely packed myofibrils and sarcoplasmic reticulum (SR) in the myotubes (Figure 2A). No T-tubules were found. After 16 days of culture highly ordered and tightly packed myofibrils can be demonstrated within the myotubes as indicated by cross-sections of intracellular structures corresponding to A- and I-bands (Figure 2B). In addition, TEM shows well developed SR (Figure 2C). Invaginations of the sarcolemma reveal the existence of T-tubules (Figure 2C). The latter finding is confirmed by SEM at higher magnification, demonstrating pits on the surface of the myotubes that represent surface openings of T-tubules (Ishikawa et al., 1983) (Figure 1D). TEM also demonstrates that SR and T-tubules form close contacts. These structures represent an equivalent of triads (Figure 2D). After 28 days of culture, signs of beginning degradation of sarcomeres together with increasing amounts of loosely packed myofibrils were detected (data not shown). Taken together, electron microscopy reveals structural features of the primary myotubes similar to those found in adult skeletal muscle.

### **3.2 Primary myotubes mainly express fast adult MHCIId/x after around three weeks of culture**

To further characterize the differentiation of the primary myotubes, the mRNA expression pattern of non-adult MHC isoforms was investigated by Northern blot analysis. For analysis of mRNA expression of MHCemb and neo, probes derived from the hypervariable 3' regions, which exhibit much more divergence than the coding regions, were used (Eller et al., 1989; Weydert et al., 1985). These regions are specific for each isoform with high sequence homology across different species (Saez and Leinwand, 1986; Moore et al., 1993; Schiaffino and Salviati, 1997). Northern blot analysis of non-adult MHC isoform mRNA in

the primary myotubes revealed a weak expression of MHCemb mRNA on day 9 (Figure 3A). On day 11, no MHCemb mRNA but significant amounts of MHCneo mRNA were found, which decreased on day 13 (Figure 3B). On day 16, MHCneo mRNA was no longer detectable (Figure 3B). Robust MHCIId/x mRNA expression was found from day 13 on, with a decrease on the level of transcription towards day 28 of culture (Figure 3C). Expression of MHCIId/x mRNA on days 14 and 21 was also demonstrated by semiquantitative RT-PCR (Figure 3D).

In accordance with the mRNA data, SDS-PAGE revealed that the primary myotubes expressed predominantly or exclusively MHCIId/x on day 13 and day 28 (Figure 4A), respectively, in line with our previous findings (Kubis et al., 1997). MHCIId/x is also the dominating MHC isoform in adult muscles from the rabbit (Aigner et al., 1993). Western blot analysis using a specific antibody against MHCIId/x also revealed an increase in the expression of MHCIId/x protein from day 14 to day 28 (Figure 4B). This finding confirms that the single band found in SDS-PAGE on day 28 indeed represents the MHC isoform IId/x. On day 21, 99% of total MHC protein expressed in the microcarrier culture was MHCIId/x, together with 1% MHCI/β. In contrast, primary myotubes cultured for 21 days on matrigel-coated culture dishes expressed 63% MHCneo together with 37% MHCIId/x (Figure 4C). With respect to the expression of adult MHC, these data demonstrate the advantage of the microcarrier culture, which obviously reach a higher stage of differentiation than the cells grown in the dishes. In the latter culture, the myotubes started to detach from around day 22 of culture. Detachment was not observed in the carrier culture around day 22 of culture, in fact the total protein content per culture flask increased until day 24 of culture, but declined towards day 29 (Figure 4D).

Taken together, the data reveal transient mRNA expression of non-adult MHC isoforms neo and emb in early phases of the primary culture, with MHCemb expression preceding MHCneo. Furthermore, coexpression of non-adult isoform mRNAs and adult MHCIId/x

mRNA was found in the intermediate stages of development. In 16 days old cultures, both non-adult MHC isoform mRNAs were no longer expressed. Therefore, it can be concluded that the primary myotubes replace non-adult by adult myosin during development as found in muscle cells *in vivo*. The predominant or exclusive expression of fast MHCIId/x indicates an adult fast type character of the primary myotubes in terms of MHC isoform pattern.

### **3.3 Increasing activity of creatine kinase indicates ongoing differentiation of the myotubes**

To further characterize the differentiation of the primary muscle cells, we determined specific creatine kinase (CK) activity (Figure 4E). CK activity increased until day 22, indicating ongoing differentiation of the myotubes. A decreasing level of CK activity on days 28 and 30 of culture compared with day 22 is in accordance with declining levels of MHCIId/x mRNA and total protein expression in the late phase of the culture (see Figures 3C and 4D), illustrating the limitations in the lifespan of the culture.

### **3.4 Cells growing on microcarriers fused to give multinucleated myotubes**

To demonstrate that the cells growing on gelatin bead microcarriers were indeed fused to multinucleated myotubes, primary myotubes grown on microcarriers for 14 days were enzymatically detached from the carriers, seeded on glass coverslips and cultured for additional two days. Staining of the nuclei of the cells with DAPI demonstrated the multinucleated character of the myotubes (Figure 5, left panel). We further used an indirect immunofluorescence approach by staining for the transcription factor NFATc1. In accordance with the demonstrated fast type character of the myotubes in terms of MHC isoform expression (Figures 3 and 4), NFATc1 immunostaining was found nearly

exclusively in the cytoplasm of the myotubes, leaving the nuclei blank (Figure 5, center panel). As demonstrated previously with the C2C12 muscle cell line (Meissner et al., 2011), addition of 0.1 mM of calcium ionophore A23187 to the medium led to nearly completely nuclear staining for NFATc1, indicating nuclear translocation (right hand panel of Figure 5). These immunofluorescence studies clearly demonstrate the multinucleated character of the myotubes growing on microcarriers.

#### 4 Discussion

The present paper demonstrates that primary rabbit myotubes grown on microcarriers in suspension develop an adult-like state with regard to their ultrastructural organisation but also with regard to the expression of MHC isoforms. TEM and SEM studies revealed structural components such as highly ordered myofibrils, T-tubules and sarcoplasmic reticulum, as they are characteristic of adult muscle cells *in vivo*. It has been demonstrated that C2C12 cells develop thin and thick filaments that are organized in myofibrils and sarcomeres after 7 to 10 d in differentiation medium (Burattini et al., 2004). But these structures do not show the same high degree of organisation as found *in vivo*. A high degree of structural differentiation has also been observed when myotubes were cocultivated with fibroblasts on Teflon membranes (Swasdison and Mayne, 1992). This culture technique yielded myofibers, which possess an ultrastructure comparable to adult fibres *in vivo*, but the special technical requirements of this method are prohibitive to using it on a larger scale. In contrast, we have demonstrated that primary skeletal muscle cells can be grown on a large scale on microcarriers for up to 30 days (Kubis et al., 1997). The major advantage of this microcarrier culture in comparison to most conventional cultures results from the way in which the myotubes grow in this system. As apparent from Figure 1, some of the myotubes grow around the surface of one carrier, while others are attached to one carrier with one end and to a second carrier with their other end. Spontaneous contractions, which begin to occur



at about one week of culturing, therefore bring the two neighbouring carriers closer to each other (see the video, supplementary file), and the contraction is more or less isotonic. But even in the case of myotubes growing on one single carrier, the contraction will not be entirely isometric, because the gelatin of the carriers is quite soft and deformable. Thus, the force exerted by the contracting myotube upon its points of attachment at the carrier/s will be much less than it is in case of a rigid support such as a conventional Petri dish, where the mode of contraction is largely isometric. The greater force exerted in the latter case causes the fibers to detach from the surface of the dish, whereupon they are lost from the culture. For this reason, the myotubes in the present culture system survive much longer and reach a more mature state than in conventional culture systems. This is clearly evident from the maturity of the ultrastructural elements as apparent from Figures 1 and 2, as well as from the purely adult pattern of MHC seen in Figures 3 and 4, which will be discussed in the following. In addition, it is conceivable that passive mechanical stretch (Goldspink et al., 1995), as exerted by the microcarriers in motion, can contribute to the differentiation of the myotubes.

The time pattern of the development of primary rabbit myotubes grown on microcarriers reveals that they reach an adult fast type II state in terms of MHC isoforms expression. As now described here on the level of mRNA (and described previously on the level of protein; Kubis et al., 1997), before day 16 of culture, a mixture of adult and non-adult MHC isoforms is expressed. However, from day 16 until day 28 exclusively adult MHC isoforms are detected on the mRNA and protein level. These data are in line with the developmental pattern of the expression of non-adult and adult MHC isoforms observed in mouse muscles *in vivo* (Buckingham, 1985; Weydert et al., 1987). During muscle development, expression of non-adult MHC isoforms precedes adult MHC isoform expression, but then also a temporary coexpression is found. Following this period of coexpression of non-adult and adult MHC isoforms, MHCIId/x is the main, and in the latest stage the sole MHC isoform

expressed in the primary culture. This agrees perfectly with the MHC expression pattern in fast muscles of adult rabbits (Aigner et al., 1993). In contrast to the microcarrier culture, primary rabbit myotubes grown on matrigel-covered culture dishes expressed substantial amounts of non-adult MHC protein. This is in agreement with Higginson et al. (Higginson et al., 2002), who observed that a primary skeletal muscle culture from rat hindleg muscle cultured on 1% matrigel-coated six-well dishes for up to 20 days did not cease to express a mixture of non-adult and adult MHC mRNA isoforms. This status of differentiation of the cultured muscle cells is also known from early studies that included different primary myotubes as well as myogenic cell lines (Silberstein et al., 1986; Weydert et al., 1987; Dusterhoft and Pette, 1993; Naumann and Pette, 1994). C2C12 cells for example have been shown to replace non-adult by adult myosin in a manner similar to that seen in the intact organism (Silberstein et al., 1986). Nevertheless, after eight days in differentiation medium, the proportion of non-adult myosin was found in the range of 25% of total myosin.

At around day 28 of culture some signs of decline of culture viability emerged, such as increased disorder of myofibrils visualized by TEM, decrease in MHCIIId/x mRNA expression, and a beginning decrease in protein content and creatine kinase activity. This may be due to lack of innervation or stimulation of the myotubes in our culture system. It remains to be determined, whether continued stimulation of the primary myotubes can increase the longevity of the culture or even accelerate the differentiation. We have demonstrated so far (Kubis et al., 1997), that electrostimulation of the primary myotubes with a slow fiber type activity pattern resulted in a fast-to-slow transformation on the level of MHC isoform expression, with the appearance of slow MHCI, comparable to what is observed when fast EDL and TA muscles are subjected to low frequency electrostimulation (Pette and Vrbová, 1992). Interestingly, only 24 h of electric pulse stimulation of multinuclear C2C12 myotubes has been reported to lead to accelerated de novo sarcomere assembly as determined by immunofluorescence (Fujita et al., 2007; Nedachi et al., 2008).

However, in this culture, expression of adult MHC isoforms was demonstrated without checking for non-adult isoforms.

In recent years, tissue engineering techniques have led to increased complexity of muscle cell culture in three-dimensional (3D) models, including vascularized skeletal muscle tissue (Levenberg et al, 2005) and co-culture of neonatal myoblasts with primary embryonic motor neurons (Martin et al., 2015). In the latter culture, a high level of striated myotubes and even the formation of neuromuscular junctions could be demonstrated. Interestingly, the co-culture also expressed a mixture of non-adult and adult myosin isoform mRNAs on day 18. This finding demonstrates that the establishment of muscle cell culture expressing exclusively adult MHC isoforms is also a challenging task in 3D models.

To summarize, primary skeletal muscle cells derived from newborn rabbit hindlimb muscles growing on gelatin bead microcarriers were characterised with respect to the time course of their differentiation. The multinucleated myotubes eventually develop features as they are found in adult skeletal muscle fibers *in vivo*. This becomes apparent on the level of ultrastructural organisation as well as of MHC isoform expression after around 16 days in culture. The myotubes then express almost exclusively fast type II MHC isoforms and can be held in culture for up to 30 days. The adult-like state of the myotubes renders them ideally suited as an *in vitro* model for the investigation of molecular interactions and mechanisms regulating fiber type-specific gene expression, for example fast-to-slow fiber-type transformations.

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## 7 Conflict of interest

None.

## 8

## References

- Aigner S, Gohlsch B, Hamalainen N, Staron RS, Uber A, Wehrle U, Pette D (1993) Fast myosin heavy chain diversity in skeletal muscles of the rabbit: heavy chain IIId, not IIb predominates. *Eur J Biochem* 211: 367-372.
- Baquero-Perez B, Kuchipudi SV, Nelli RK, Chang KC (2012) A simplified but robust method for the isolation of avian and mammalian muscle satellite cells. *BMC Cell Biol* 13: 16.
- Bass A, Brdiczka D, Eyer P, Hofer S, Pette D (1969) Metabolic differentiation of distinct muscle types at the level of enzymatic organization. *Eur J Biochem* 10: 198-206.
- Bassam BJ, Caetano-Anolles G, Gresshoff PM (1991) Fast and sensitive silver staining of DNA in polyacrylamide gels. *Anal Biochem* 196: 80-83.
- Booth FW, Baldwin KM (1996) Muscle plasticity: energy demand and supply processes. In: Rowell LB, Shepherd JT, editors. *Handbook of Physiology, Section 12: Exercise: Regulation and Integration of Multiple Systems*. Oxford: Oxford University Press, pp 1075-1123.
- Buckingham ME (1985) Actin and myosin multigene families: their expression during the formation of skeletal muscle. *Essays Biochem* 20: 77-109.
- Burattini S, Ferri P, Battistelli M, Curci R, Luchetti F, Falcieri E (2004) C2C12 murine myoblasts as a model of skeletal muscle development: morpho-functional characterization. *Eur J Histochem* 48: 223-233
- Cannon-Carlson S, Tang J (1997) Modification of the Laemmli sodium dodecyl sulfate-polyacrylamide gel electrophoresis procedure to eliminate artifacts on reducing and nonreducing gels. *Anal Biochem* 246: 146-148.
- Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156-159.

- Dusterhoft S, Pette D (1993) Satellite cells from slow rat muscle express slow myosin under appropriate culture conditions. *Differentiation* 53: 25-33.
- Eisenberg BR (1983) Quantitative ultrastructure of mammalian skeletal muscle. In: Peachey LD, editor. *Handbook of Physiology, Section 10: Skeletal Muscle*. Bethesda, Maryland: American Physiological Society, pp 73-112.
- Eller M, Stedman HH, Sylvester JE, Fertels SH, Wu QL, Raychowdhury MK, Rubinstein NA, Kelly AM, Sarkar S (1989) Human embryonic myosin heavy chain cDNA. Interspecies sequence conservation of the myosin rod, chromosomal locus and isoform specific transcription of the gene. *FEBS Lett* 256: 21-28.
- Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 132: 6-13.
- Fluck M, Hoppeler H (2003) Molecular basis of skeletal muscle plasticity-from gene to form and function. *Rev Physiol Biochem Pharmacol* 146: 159-216.
- Fujita H, Nedachi T, Kanzaki M (2007) Accelerated de novo sarcomere assembly by electric pulse stimulation in C2C12 myotubes. *Exp Cell Res* 313: 1853-1865.
- Goldspink DF, Cox VM, Smith SK, Eaves LA, Osbaldeston NJ, Lee, DM, Mantle (1995) Muscle growth in response to mechanical stimuli. *Am J Physiol* 268: E288-E297.
- Goldspink G (2002) Gene expression in skeletal muscle. *Biochem Soc Trans* 30: 285-290.
- Heukeshoven J, Dernick R (1985) Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. *Electrophoresis* 6: 103-112.
- Higginson J, Wackerhage H, Woods N, Schjerling P, Ratkevicius A, Grunnet N, Quistorff B (2002) Blockades of mitogen-activated protein kinase and calcineurin both change fibre-type markers in skeletal muscle culture. *Pflugers Arch* 445: 437-443.
- Ishikawa H, Sawada H, Yamada E (1983) Surface and internal morphology of skeletal muscle. In: Peachey LD, editor. *Handbook of Physiology, Section 10: Skeletal Muscle*. Bethesda, Maryland: American Physiological Society, pp 1-22.
- Katz RA, Erlanger BF, Guntaka RV (1983) Evidence for extensive methylation of ribosomal RNA genes in a rat XC cell line. *Biochim Biophys Acta* 739: 258-264.
- Kubis, HP, Gros, G (1997) A rapid electrophoretic method for separating rabbit skeletal muscle myosin heavy chains at high resolution. *Electrophoresis* 18: 64-66.
- Kubis HP, Haller EA, Wetzel P, Gros G (1997) Adult fast myosin pattern and Ca<sup>2+</sup>-induced slow myosin pattern in primary skeletal muscle culture. *Proc Natl Acad Sci U S A* 94: 4205-4210.

- Levenberg S, Rouwkema J, Macdonald M, Garfein ES, Kohane DS, Darland DC, Marini R, van Blitterswijk CA, Mulligan RC, D'Amore PA, Langer R (2005) Engineering vascularized skeletal muscle tissue. *Nat Biotechnol* 23:879-84.
- Maeda K, Sczakiel G, Wittinghofer A (1987) Characterization of cDNA coding for the complete light meromyosin portion of a rabbit fast skeletal muscle myosin heavy chain. *Eur J Biochem* 167: 97-102.
- Martin NR, Passey SL, Player DJ, Mudera V, Baar K, Greensmith L, Lewis MP (2015) Neuromuscular junction formation in tissue-engineered skeletal muscle augments contractile function and improves cytoskeletal organization. *Tissue Eng Part A* 21: 1-10.
- McCullagh KJ, Calabria E, Pallafacchina G, Ciciliot S, Serrano AL, Argentini C, Kalhovde JM, Lomo T, Schiaffino S (2004) NFAT is a nerve activity sensor in skeletal muscle and controls activity-dependent myosin switching. *Proc Natl Acad Sci U S A* 101: 10590-10595.
- Meissner JD, Gros G, Scheibe RJ, Scholz M, Kubis HP (2001) Calcineurin regulates slow myosin, but not fast myosin or metabolic enzymes, during fast-to-slow transformation in rabbit skeletal muscle cell culture. *J Physiol* 533: 215-226.
- Meissner JD, Freund R, Krone D, Umeda PK, Chang K-C, Gros G, Scheibe RJ (2011) Extracellular signal-regulated kinase 1/2-mediated phosphorylation of p300 enhances myosin heavy chain I/ $\beta$  gene expression via acetylation of nuclear factor of activated T cells c1. *Nucleic Acids Res* 39: 5907-5925.
- Moore LA, Tidymann WE, Arrizubieta MJ, Bandman E (1993) The evolutionary relationship of avian and mammalian myosin heavy-chain genes. *J Mol Evol* 36: 21-30.
- Naumann K, Pette D (1994) Effects of chronic stimulation with different impulse patterns on the expression of myosin isoforms in rat myotube cultures. *Differentiation* 55: 203-211.
- Nedachi T, Fujita H, Kanzaki M (2008) Contractile C2C12 myotube model for studying exercise-inducible responses in skeletal muscle. *Am J Physiol Endocrinol Metab* 295: E1191-E1204.
- Oyama VJ, Eagle E (1956) Measurement of cell growth in tissue culture with a phenol reagent (folin-ciocalteu). *Proc Soc Exp Biol Med* 91: 305-307.
- Peachey LD, Franzini-Armstrong C (1983) Structure and function of membrane systems in skeletal muscle cells. In: Peachey LD, editor. *Handbook of Physiology, Section 10: Skeletal Muscle*. Bethesda, Maryland: American Physiological Society, pp 23-72.
- Pette D, Vrbová G (1992) Adaptation of mammalian skeletal muscle fibers to chronic electrical stimulation. *Rev Physiol Biochem Pharmacol* 120: 115-202.

- Pette D, Vrbova G (1999) What does chronic electrical stimulation teach us about muscle plasticity? *Muscle Nerve* 22: 666-677.
- Saez L, Leinwand LA (1986) Characterization of diverse forms of myosin heavy chain expressed in adult human skeletal muscle. *Nucleic Acids Res* 14: 2951-2969.
- Scharf M, Neef S, Freund R, Geers-Knorr C, Franz-Wachtel M, Brandis A, Krone D, Schneider H, Groos S, Menon MB, Chang KC, Kraft T, Meissner JD, Boheler KR, Maier LS, Gaestel M, Scheibe RJ (2013) Mitogen-activated protein kinase-activated protein kinases 2 and 3 regulate SERCA2a expression and fiber type composition to modulate skeletal muscle and cardiomyocyte function. *Mol Cell Biol* 33: 2586-2602.
- Schiaffino S (2010) Fibre types in skeletal muscle: a personal account. *Acta Physiol (Oxf)* 199: 451-463.
- Schiaffino S, Salviati G (1997) Molecular diversity of myofibrillar proteins: isoforms analysis at the protein and mRNA level. *Methods Cell Biol* 52: 349-369.
- Serrano AL, Murgia M, Pallafacchina G, Calabria E, Coniglio P, Lomo T, Schiaffino S (2001) Calcineurin controls nerve activity-dependent specification of slow skeletal muscle fibers but not muscle growth. *Proc Natl Acad Sci U S A* 98: 13108-13113.
- Silberstein L, Webster SG, Travis M, Blau HM (1986) Developmental progression of myosin gene expression in cultured muscle cells. *Cell* 46: 1075-1081.
- Swadison S, Mayne R (1992) Formation of highly organized skeletal muscle fibers in vitro. Comparison with muscle development in vivo. *J Cell Sci* 102: 643-652.
- Uber A, Pette D (1993) PCR-based assignment of two myosin heavy chain cDNA clones to biochemically and histochemically defined single type IIB and IID fibers of rabbit muscle. *FEBS Lett* 331: 193-197
- Vandeburgh HH, Swadison S, Karlisch P (1991) Computer-aided mechanogenesis of skeletal muscle organs from single cells in vitro. *FASEB J* 5: 2860-2867.
- Wehrle U, Dusterhoft S, Pette D (1994) Effects of chronic electrical stimulation on myosin heavy chain expression in satellite cell cultures derived from rat muscles of different fiber-type composition. *Differentiation* 58: 37-46.
- Weydert A, Barton P, Harris AJ, Pinset C, Buckingham M (1987) Developmental pattern of mouse skeletal myosin heavy chain gene transcripts in vivo and in vitro. *Cell* 49: 121-129.
- Weydert A, Daubas P, Lazaridis I, Barton P, Garner I, Leader DP, Bonhomme F, Catalan J, Simon D, Guenet JL, Gros F, Buckingham ME (1985) Genes for skeletal muscle myosin heavy chains are clustered and are

not located on the same mouse chromosome as a cardiac myosin heavy chain gene. Proc Natl Acad Sci U S A 82: 7183-7187.

Zebedin E, Sandtner W, Galler S, Szendroedi J, Just H, Todt H, Hilber K (2004) Fiber type conversion alters inactivation of voltage-dependent sodium currents in murine C2C12 skeletal muscle cells. Am J Physiol Cell Physiol 287: C270-C280.

Zetser A, Gredinger E, Bengal E (1999) p38 mitogen-activated protein kinase pathway promotes skeletal muscle differentiation. Participation of the Mef2c transcription factor. J Biol Chem 274: 5193-5200.

## 9 Figure legends

**9.1 Figure 1 Scanning electron microscopy (SEM) reveals structural features of primary rabbit myotubes as found in adult skeletal muscle.** Scanning electron micrographs of primary myotubes grown on microcarriers (A) for 9 days, light bar: 0.1 mm, (B) and (C) for 16 days, light bar: 0.1 mm, and **d** 16 days, light bar: 10  $\mu$ m. T: T-tubule, indicated by arrows.

**9.2 Figure 2 Transmission electron microscopy (TEM) reveals structural features of primary rabbit myotubes as found in adult skeletal muscle.** Transmission electron micrographs of myotubes grown on microcarriers for (A) 9 days, (B), (C) and (D) for 16 days, black bar: 1  $\mu$ m. A: A-band; G: glycogen granules, indicated by an arrow; I: I-band, Mf: myofibrils; Mi: mitochondria; SL: sarcolemm, indicated by an arrow; SR: sarcoplasmic reticulum, indicated by an arrow; T: T-tubule, indicated by an arrow; Tr: triad, indicated by an arrow; Z: Z-disc, indicated by an arrow.

**9.3 Figure 3 Primary rabbit myotubes no longer express non-adult MHC isoform mRNA after 16 days of culture.** Northern blot analysis of the developmental pattern of MHC isoform mRNA expression. Primary myotubes were grown on microcarriers for 9, 11, 13, 16, 21, or 28 days as indicated. Total RNA (20  $\mu$ g) was isolated, fractionated on a



formaldehyde agarose gel, transferred to nitrocellulose, and probed (A) with the 3' terminal *Bam*HI/*Eco*RI fragment of embryonic MHC cDNA (MHCemb), or 18S rDNA, (B) with the 3' terminal *Pst*I fragment of neonatal MHC cDNA (MHCneo), or 18S rDNA, (C) with the 3' terminal *Pst*I fragment of adult fast MHCIId/x cDNA, or 18S rDNA. Probes were [<sup>32</sup>P]-labeled. The position of 18S rRNA and 28S rRNA on the ethidium bromide-stained gels are indicated. (D) Semiquantitative RT-PCR analysis of MHCIId/x mRNA expression. Total RNA was isolated from primary rabbit myotubes grown on microcarriers for 14 or 21 days. Densitometric quantification of PCR products separated by PAGE and visualized with highly sensitive silver staining. 18s rRNA levels were used for normalisation.

**9.4 Figure 4 Primary rabbit myotubes grown on microcarrier predominantly express fast adult MHCII protein after three weeks of culture.** (A) Gelelectrophoretic analysis of MHC isoform expression in myotubes growing on microcarriers. SDS-PAGE of myosin extracts at days 13 and 28 of culture. M: Marker, mixture of myosin extracts from rabbit soleus (MHCI/β and IIa) and tibialis anterior (MHCIIa and IId/x) muscle, emb: embryonic MHC, neo: neonatal MHC. (B) Western blot analysis demonstrates increased expression of MHCIId/x protein from day 14 to day 28. Primary rabbit myotubes were grown on microcarriers for 14 or 28 days. The blot was probed with an anti-MHCIId/x antibody and reprobed with anti-α-tubulin antibody as loading control. The position of marker proteins is shown on the left. (C) Quantification of SDS-PAGE analysis of myosin extracts from primary myotubes grown on microcarriers or matrigel-coated culture dishes at day 21 of culture. Total MHC band density per cultivation method was set to 100%. (D) Total cell protein in primary rabbit muscle cells grown on microcarriers increased until day 24 of culture. Cell protein per culture flask (mg) was determined at different age of culture (6, 11, 14, 16, 21, 24, and 28 days). The data represent the mean ± SD, n ≥ 3. \*: significantly different from day 6, *p* < 0.05; +: significantly different from day 24, *p* < 0.05. (E) Increasing

activity of creatine kinase indicates ongoing differentiation of primary rabbit myotubes. Time course of specific creatine kinase activity of primary muscle cells grown on microcarriers. Creatine kinase activity (units per g protein, U/g PRT) was determined at different age of culture (9, 11, 16, 22, 28 and 30 days). The data represent the mean  $\pm$  SD,  $n=3$ . \*: significantly different from day 9,  $p<0.05$ ; +: significantly different from day 22,  $p<0.05$ .

#### **9.5 Figure 5 Cells growing on microcarriers fused to give multinucleated myotubes.**

Primary myotubes grown on microcarriers for 14 days were enzymatically detached from the carriers and grown on glass coverslips for additional two days in DM or for one day in DM and for additional one day in DM in the presence or absence of 0.1 mM  $\text{Ca}^{2+}$ -ionophore A23187 (Ionophor) as indicated. Myotubes were stained with DNA-marker DAPI to depict the nuclei or processed for immunofluorescence staining using a primary anti-NFATc1 and a FITC-labeled secondary antibody as indicated. Fluorescence was detected by using an inverted fluorescence photomicroscope at a magnification of x 400.

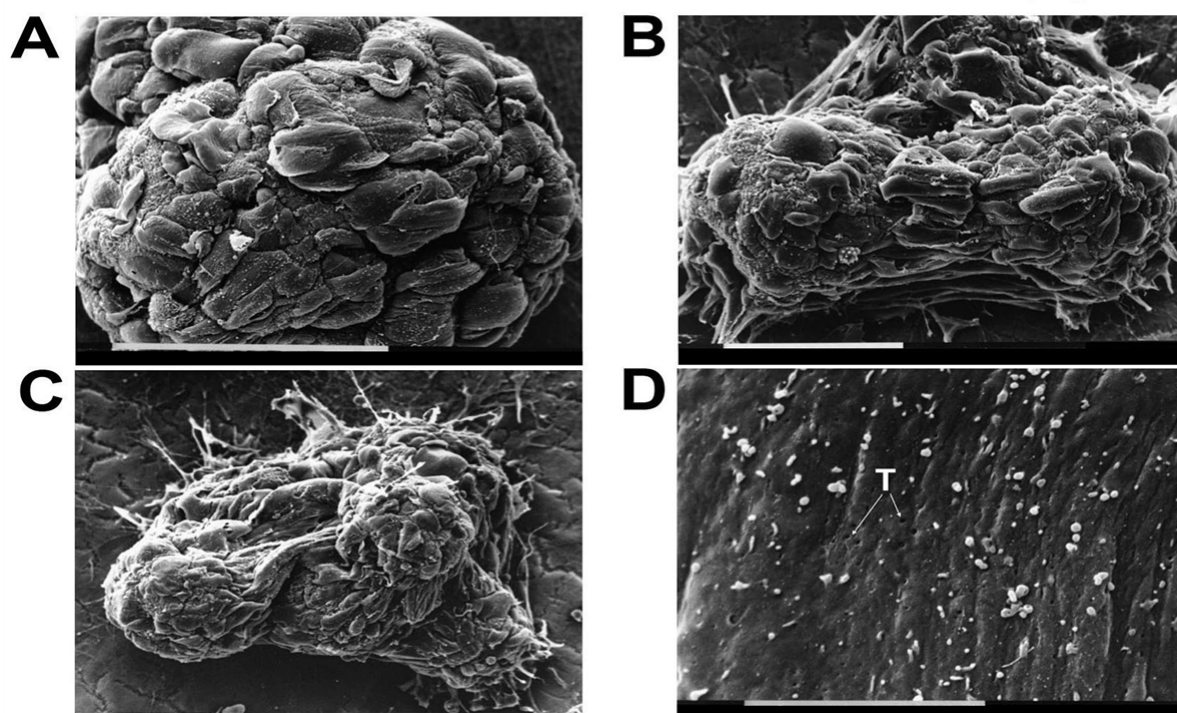


Figure 1.

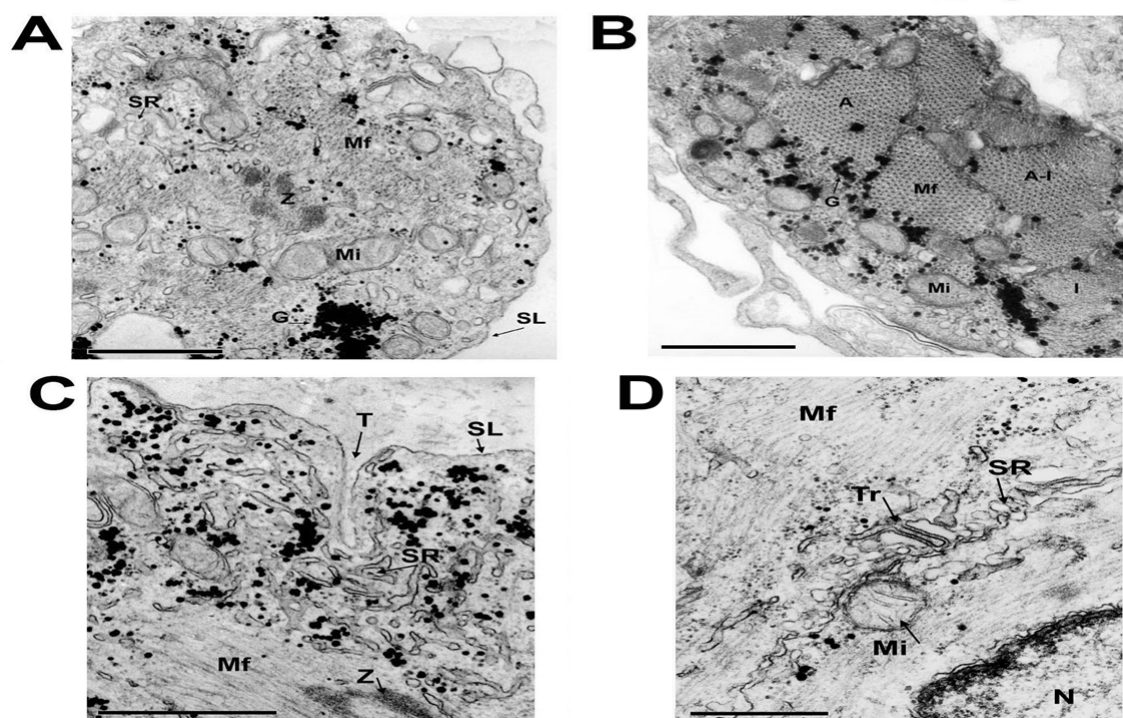


Figure 2.

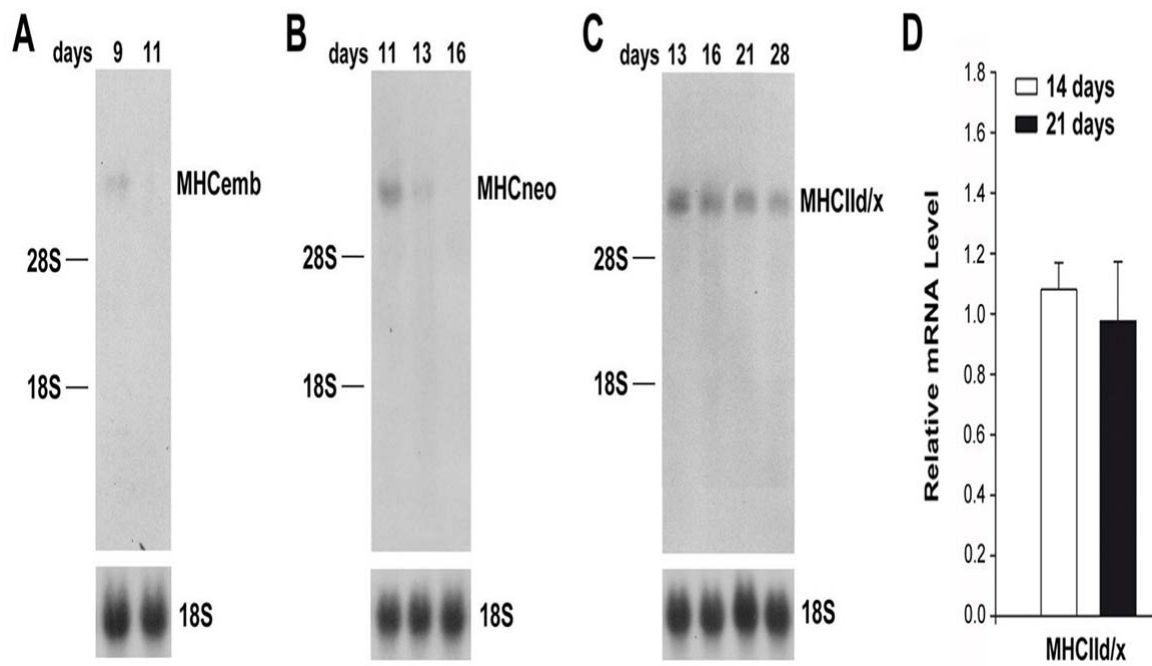


Figure 3.

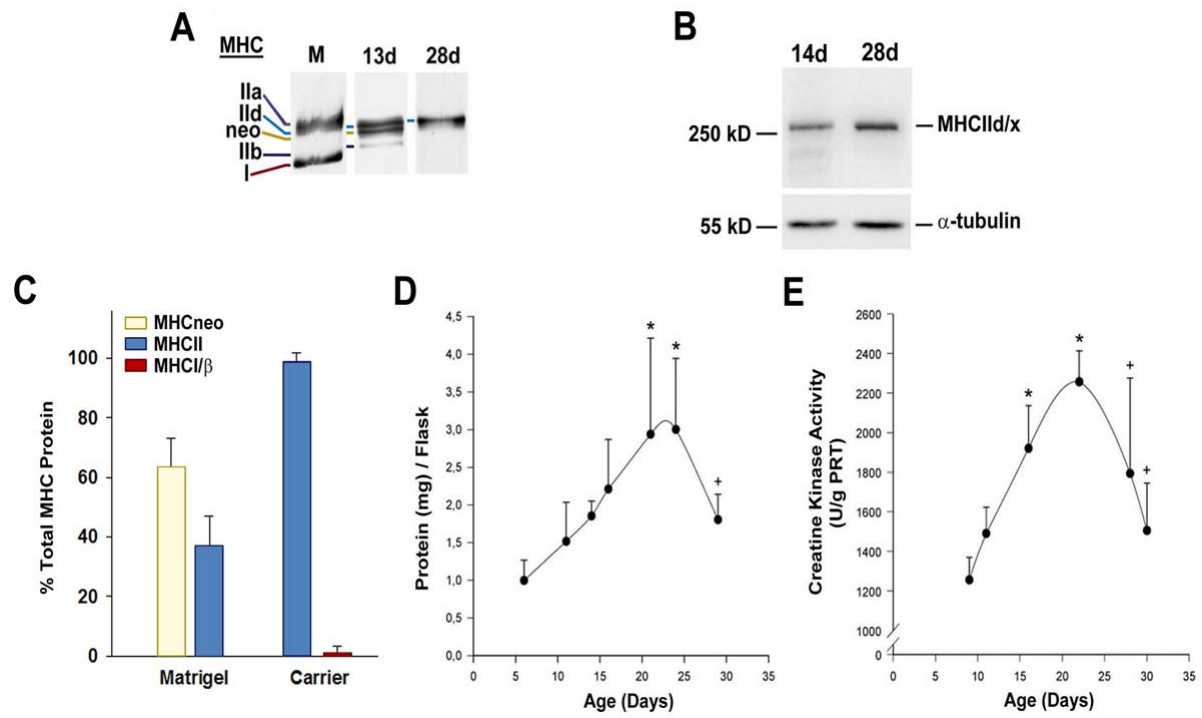


Figure 4.

## Kubis et al.\_Figure 5

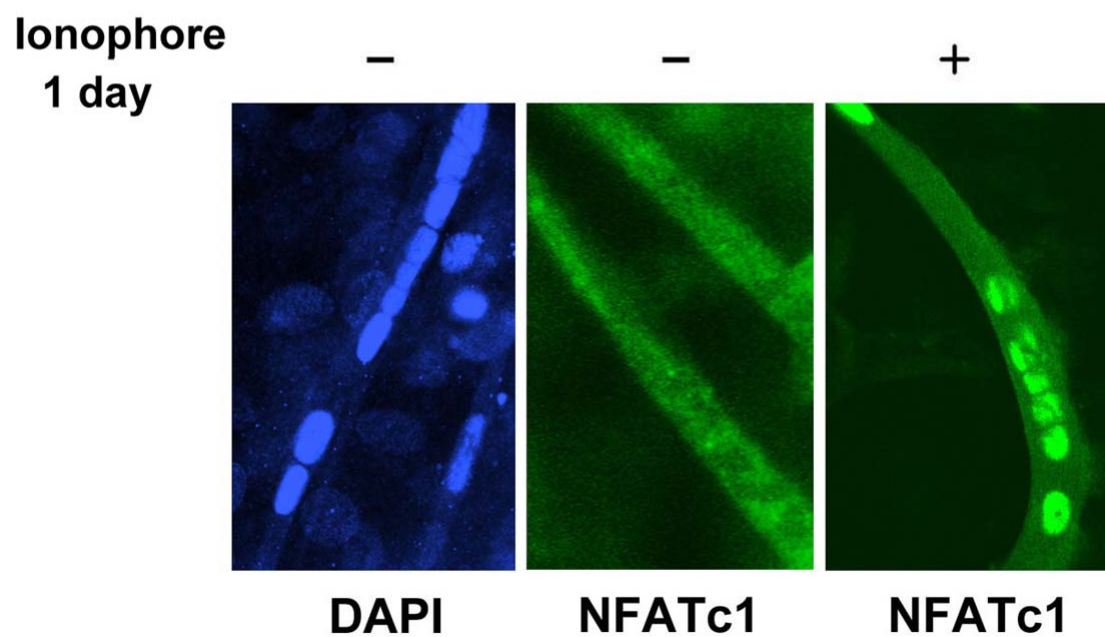


Figure 5.